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Cytokine single nucleotide polymorphisms and intrarenal gene expression in chronic allograft nephropathy in children

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Background. Single nucleotide polymorphisms (SNPs) have been reported to influence cytokine production. Certain cytokine high producer genotypes have been associated with an increased risk for acute rejection and chronic allograft dysfunction (CAD) after transplantation. Our study evaluates SNP distribution for transforming growth factor-β1 (TGF-β1), interleukin-10 (IL-10), and tumor necrosis factor-α (TNF-α) in pediatric renal transplant recipients and control individuals and correlates them to corresponding intrarenal gene expression.

Methods. SNPs for TGF-β1 (codon 10 and 25), IL-10 (positions –1082, −819, −592) and TNF-α (position −308) were determined in 30 patients with stable graft function, in 75 patients with CAD, and in 173 control individuals by allele-specific polymerase chain reaction (PCR). Intrarenal cytokine gene expression was studied in 25 biopsies with chronic allograft nephropathy (CAN) and 27 normal kidney specimens by real-time reverse transcription (RT)-PCR.

Results. No difference in allele and genotype frequency was detected in any of the investigated groups. No correlation between genotype and intragraft cytokine gene expression was recognized in CAN patients. However, in normal kidney specimens, the low producer TGF-β1 genotype at codon 10 was associated with significant lower TGF-β1 mRNA expression. This association was not found for IL-10 or TNF-α.

Conclusion. Our results do not support the proposition that certain specific cytokine genotypes for TGF-β1, IL-10, and TNF-α are associated with CAD. Intrarenal cytokine gene expression only correlated to one TGF-β1 SNP in normal kidney specimens. Since overall TGF-β1 expression was higher in transplanted patients compared to controls, this suggests that SNPs may not play a significant role once the immune system is activated.

Key words: TGF-β1, IL-10, TNF-α, single nucleotide polymorphisms, chronic allograft nephropathy, pediatric transplantation.

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In recent years single nucleotide polymorphisms (SNPs) have become of increasing interest because of their possible association with certain disease states. In transplantation, SNPs in cytokine genes have been reported to indicate a higher risk for acute rejection and chronic allograft dysfunction (CAD) by several groups [1, 2]. Genotyping of transforming growth factor-β1 (TGF-β1), interleukin-10 (IL-10), and tumor necrosis factor-α (TNF-α) SNPs has been suggested in order to establish predictive markers for the posttransplant course in individual patients.

TGF-β is a multifactorial cytokine that initiates and terminates tissue repair and its sustained production regulates the development of tissue fibrosis [3]. It has also been associated with the promotion of renal allograft interstitial fibrosis and thereby chronic allograft nephropathy (CAN) [4, 5]. Two SNPs in the first exon of TGF-β1, the best evaluated isoform of this gene, have been described at position +869 (T/C) and at position +915 (G/C). These polymorphisms result in the change of codon 10 from leucine (T) to proline (C) and in the change of codon 25 from arginine (G) to proline (C) [6]. In vitro, the presence of leucine or arginine, respectively, has been shown to lead to a higher production of TGF-β1 [1, 7].

IL-10 is an anti-inflammatory cytokine but may promote antibody responses by its stimulatory effect on B cells. Three SNPs in the promotor region of IL-10 are most commonly studied in the context of transplantation: Positions –1082 (G/A), –819 (C/T) and –592 (C/A) upstream from the transcriptional start site [8]. The presence of an "A" at −1082 and at −592 has been related to low IL-10 production in cultured cells [7–10]. This low producer allele has been correlated with increased incidence of rejection in heart and kidney graft recipients in some studies [11, 12] but not in others [13–15]. In fact, one group described the presence of high-producing IL-10 genotype as a risk factor for higher incidence and severity of acute rejection [15]. The same group found that the high-producing IL-10 genotype might have long-term protection in kidney transplantation [16].

TNF-α is a potent proinflammatory cytokine. Most commonly studied is the SNP at −308 that involves a G/A substitution [17]. It has been shown that an "A" at position –308 was associated with increased transcription and production of TNF-α [18, 19]. Patients with either renal or cardiac transplants and the high-producing TNF-α genotype were found to be at increased risk for rejection [11, 14, 15, 20].

The literature on cytokine polymorphisms and their importance on long-term graft survival is heterogeneous. In addition, the relationship between cytokine SNPs and cytokine production is based on in vitro studies. Apart from a single case report on two patients [21], no study has tried to combine SNP cytokine data with intragraft cytokine expression. In the present study, our aim was to evaluate the significance of polymorphisms in the coding region of TGF-β1 and in the promotor regions of IL-10 and TNF-α for long-term graft survival. We assessed whether the so-called high-producing genotypes are more frequent in our pediatric patient collective with CAD as compared to patients with stable graft function or normal control subjects. Furthermore, in vivo intrarenal cytokine production in transplant biopsies with CAN and normal control kidney specimens was compared to the underlying genotype.

METHODS

Patients

All recipients of renal transplants presenting to the outpatient clinic (Departments of Pediatric Nephrology, Hannover Medical School, Hannover, and Charité, Berlin, Germany) between May 1999 and April 2000 were asked to participate and follow-up was performed until July 2002. Of these patients, 105 were selected for this study: 30 patients with stable graft function, which was defined as no or less than five percent change in their glomerular filtration rate (GFR) per year, and 75 patients with chronic deterioration of their graft function [chronic allograft dysfunction (CAD)], including 42 with biospy-proven CAN. Patients had been transplanted between April 1983 and May 1999, with mean follow-up time of 8.9 (±4.4) years for stable and 8.5 (±3.6) years for CAD. Sixty-six (stable, $N = 16$; CAD, $N = 50$) patients received a cadaveric transplant and 39 (stable, $N = 14$; CAD, $N = 25$) patients had a living related donor transplantation. At the time of transplantation, the patients were between 1 to 16 years old (stable, 8.6 ± 4.4 years; CAD, 9.2 ± 4.3 years) and 54% were male recipients (stable, $N = 14$ males and 16 females; CAD, $N = 43$ males and 32 females). All patients were on a similar immunosuppressive regimen, including cyclosporine A (adjusted to a target through level of 150 ± 50 ng/mL) and prednisolone (4 mg/m^2) and azathioprine (2 mg/kg). After CAN-confirming biopsy, 40 patients were switched to mycophenolate mofetil as rescue therapy (abstract; Henne et al, *Am J Transplant* 1: 150, 2001). Blood for cytokine polymorphism typing was taken at any time after transplantation. Snap-frozen sections of renal biopsies had been collected prospectively at one of the participating centers (Hannover Medical School, Hannover) between January 1997 and December 1999 for gene expression analysis. We were able to determine intragraft cytokine expression in 25 biopsies diagnosed with CAN.

Controls

Umbilical cord blood was used for determination of cytokine polymorphisms (Cpoly) in 173 newborns born at the neonatal unit of Hannover Medical School, Hannover, to serve as controls for distribution of polymorphisms in Germany.

Twenty-eight renal cortex specimens (Cex) derived from nephrectomies due to renal cell carcinoma served as controls for intrarenal cytokine expression [22, 23]. Macroscopically tumor-free kidney was used to separate cortex from medulla and samples were snap-frozen in liquid nitrogen prior to storage and subsequent RNA extraction. Sections from immediate vicinity of these samples were taken for routine histology, stained, and reviewed for the absence of neoplasma, inflammation, and changes greater than expected with age [22].

Ethics approval

The study protocol was approved by the Institutional Review Board of the Hannover Medical School, Hannover, Germany (Decision #2054).

Cytokine polymorphisms analysis

Genomic DNA from whole blood or renal tissue was obtained using the DNeasy Kit (QIAGEN, Hilden, Germany) following the instructions of the manufacturer. All samples were analyzed for two polymorphisms in the TGF-β1 coding region at position +869 (codon 10) and +915 (codon 25), for three polymorphisms in the IL-10 promotor region at position −1082, −819 and −592 and for one polymorphism in the TNF-α promotor region at position −308. For detection of all polymorphisms, the Cytokine Typing PCR-SSP Tray Kit (Collaborative Transplant Study, Heidelberg, Germany) was used according to the manufacturer's instructions. Cycling conditions were as follows: 94°C for 2 minutes; 10 cycles at 94°C for 10 seconds, and 65°C for 1 minute; 20 cycles at 94°C for 10 seconds, 61°C for 50 seconds, and 72°C for 30 seconds. All polymerase chain reactions (PCRs) were performed on a Perkin Elmer 9600 Thermal Cycler (Perkin Elmer, Rodgau, Germany).

Cytokine expression analysis

Ten percent of a core biopsy cylinder was cut and snap-frozen in liquid nitrogen. Total RNA was extracted

using the RNeasy Kit (QIAGEN) after initial homogenization. Reverse transcription (RT) was performed using random hexamer primers (Roche, Mannheim, Germany) and Moloney-murine leukemia virus (MMLV) (Life Technologies, Gibco BRL, Eggenstein, Germany) as previously described [23]. Quantitative real-time PCR to detect TGF-β1, IL-10, and TNF-α gene expression was performed on an ABIPrism SDS 7700 TaqMan (Applied Biosystems, Weiterstadt, Germany) using predeveloped assay reagents (PDAR) (Applied Biosystems) according to the standard protocol supplied by the manufacturer. All samples were measured in triplicate. Positive and negative controls were included with each run. Relative quantification of gene expression was performed by normalization to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) after determining the first cycle of fluorescent detection (threshold cycle) and calculating the differences of these threshold cycles (delta threshold cycle method) as described earlier [23].

Statistical analysis

Statistical analysis was performed using SPSS. For comparison of allele and genotype frequencies the chi-square test was used. The level of significance was corrected for the number of SNP examined for each cytokine (Bonferroni correction for multiple comparisons). Cytokine expression was compared using Mann-Whitney U test for comparison of two and Kruskal-Wallis test for comparison of three groups.

RESULTS

SNPs for TGF-β1, IL-10, and TNF-α

High-producing phenotypes have been suggested for TGF-β1 in case of the homozygous presence of the T allele at codon 10 or the G allele at codon 25; for IL-10 in case of homozygosity for G, C, and C alleles at positions −1082, −819, and –592, respectively; and for TNF-α in case of the A allele at position −308. For TGF-β1 and IL-10, for which we analyzed more than one SNP, this results in the following high-producer haplotypes: TG/TG and GCC/GCC.

Allelic distribution for TGF-β1 showed that two thirds of normal individuals (63%, $N = 217$) had the T allele at codon 10 and 94% ($N = 324$) had the G allele at codon 25. For IL-10 at position –1082, the G allele was found in 40% ($N = 137$) of normal control individuals. As previously described [13, 24], SNPs at position −819 and –592 for IL-10 were linked in all individuals leading to CC and TA haplotypes, respectively. Seventy-five percent ($N = 261$) of control individuals had the CC haplotype. The A allele for TNF-α was found in 15% ($N = 51$) of control individuals. The similar allelic distributions were seen in patients with CAD and stable patients: TGF-β1, T allele in 65% ($N = 97$) of CAD and in 63% ($N = 38$) of stable and G allele in 95% ($N = 143$) of CAD and in 95% ($N = 57$) of stable; IL-10, G allele in 39% ($N = 58$) of CAD and in 43% ($N = 26$) of stable and C alleles in 63% ($N = 95$) of CAD and in 72% ($N = 43$) of stable; TNF-α, A allele in 17% ($N = 26$) of CAD and in 15% ($N = 9$) of stable. In particular, there was no significant difference between CAD and stable patients.

Table 1. Absolute numbers and percentages for transforming growth factor-β1 (TGF-β1), interleukin 10 (IL-10) and tumor necrosis factor-α (TNF-α) genotypes frequencies in pediatric renal transplant recipients with stable function and chronic allograft dysfunction (CAD) and control individuals

	Cpoly		Stable		CAD		Cex	
Alleles	*N*	%	*N*	%	*N*	%	*N*	%
TGF-β1								
codon 10								
TT	67	39	8	27	29	39	6	21
TC	83	48	22	73	39	52	16	58
CC	23	13	0	0	7	9	6	21
codon 25								
GG	151	87	27	90	68	91	23	82
GC	22	13	3	10	7	9	5	18
CC	0	0	0	0	0	0	0	0
IL-10								
−1082								
GG	28	16	7	23	8	11	4	14
GA	81	47	12	40	42	56	18	64
AA	64	37	11	37	25	33	6	22
−819 and −592								
CC and CC	97	56	17	57	31	41	15	53
CT and CA	67	39	9	30	33	44	10	36
TT and AA	9	5	4	13	11	15	3	11
TNF-α								
AA	10	6	1	3	1	1	2	7
AG	31	18	7	23	24	32	9	32
GG	132	76	22	74	50	67	17	61

Abbreviations are: Cpoly, control group for single nucleotide polymorphisms (SNPs) cytokines; Cex, control group for cytokine expression. No statistically significant differences occurred.

We analyzed the genotype distribution for the three cytokines and corresponding six SNPs (Table 1). For TGF-β1 codon 10, most individuals were either homozygous or heterozygous for the T allele (i.e., most individuals should show either a high or an intermediate producer phenotype). When distribution for stable and CAD patients was compared with normal controls and to each other, no significant differences were observed. However, none of the stable patients had homozygous presence of the C allele at TGF-β1 codon 10 that would result in a low-producing phenotype. Genotypes for TGF-β1 codon 25 were either homozygous (GG) or heterozygous (GC) for G with the homozygous genotype being most common (Cpoly, 87%). The homozygous genotype CC was not found in any group. Comparison of all groups revealed no significant differences. In the case of the IL-10 SNP at –1082, the majority of normal control individuals turned out to be heterozygous (GA = 47%) followed by being homozygous for A (37%) and for G (16%). The same ranking was found when we looked at

stable and CAD patients. However, the CAD patient group had the lowest percentage of homozygous GG genotype (11% as compared to 16% in Cpoly and 23% in stable) that would be associated with high-producing phenotype. None of the comparisons reached statistical significance. Most control individuals were homozygous (CC and CC = 56%) or heterozygous (CT and CA = 39%) for the IL-10 alleles at positions −819 and –592. The similar distribution was seen for stable and CAD patients, with CAD patients having the highest percentage of TT and AA genotypes (15% as compared to 5% in Cpoly and 13% in stable) that would result in low IL-10 production. None of these slight differences were statistical significant after correction for multiple comparisons. Control individuals presented with either a TNF-α homozygous (76%) or heterozygous (18%) G allele at position –308, the homozygous presence of A was rare (6%). Comparison of both patients groups with the controls and with each other showed the similar distribution pattern and no significant differences. Most CAD patients belonged to the group of intermediate producers.

Haplotype frequencies for TGF-β1 and IL-10 were analyzed accordingly (data not shown) and no significant differences were found.

Intrarenal cytokine expression of TGF-β1, IL-10, and TNF-α

Intrarenal cytokine expression was measured by quantitative real time PCR in 25 graft biopsies of patients with CAN and 28 normal renal Cex.

Allelic and genotype distribution for this second control group was similar to the other groups (Table 1) with no significant differences detected. Even though we did not find any differences regarding their genotypic profile, we analyzed cytokine expression in the patient and control group separately.

Overall cytokine expression was different between the CAN patients and normal controls (Figs. 1, 2, and 3). In CAN patients, TGF-β1 and TNF-α expression was significantly higher (1.6- and threefold, $P < 0.05$, respectively) and IL-10 expression was significantly lower (2.9-fold, $P < 0.05$) when compared to normal controls.

We then analyzed the expression levels according to the underlying genotype. In patients with CAN, TGF-β1 mRNA levels were not associated with the underlying genotypes for either of the two TGF-β1 SNPs at codon 10 and codon 25 as illustrated in Figure 1. However, in normal controls we found significant higher TGF-β1 levels for the high (TT) compared to the low (CC) producer polymorphism at codon 10 ($P < 0.01$). No differences were seen for codon 25.

We were unable to find matching intrarenal cytokine production as suggested by their predicted phenotype for any of the investigated IL-10 genotypes (Fig. 2). CAN

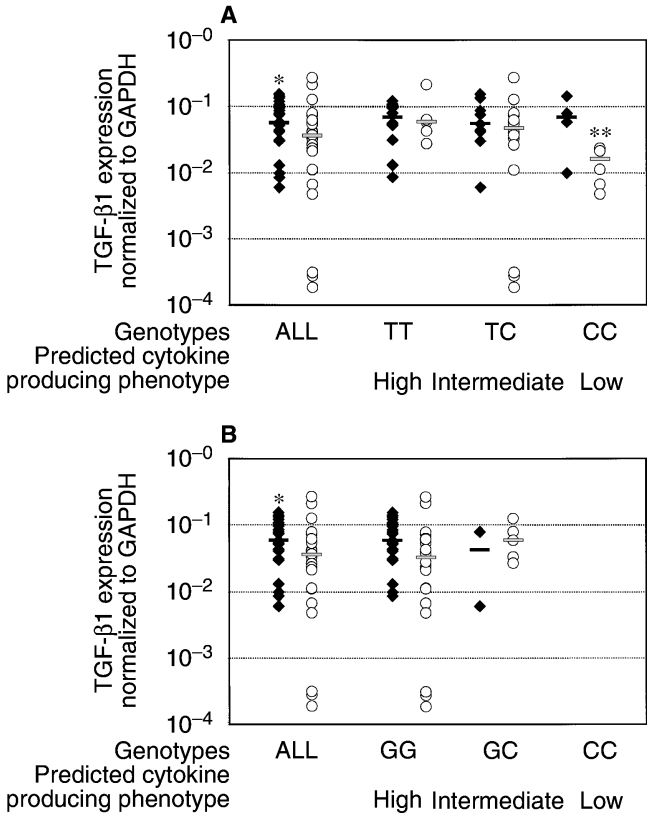

Fig. 1. Intrarenal transforming growth factor (TGF-β1) mRNA expression for pediatric renal transplant recipients with chronic allograft nephropathy (CAN) ($N = 25$) (◆) and control individuals ($N = 28$) (○) for TGF-β1 codon 10 (*A*) and TGF-β1 codon 25 (*B*). Overall (ALL) TGF-β1 expression was different between CAN and controls. $*P < 0.05$ for the comparison with controls. However, for the different TGF-β1 genotypes and their predicted producing phenotypes (High, Intermediate, Low) only the comparison between Low vs. High for codon 10 in controls resulted in a significant difference. $**P < 0.001$ for the comparison with the TT genotype). GAPDH is glyceraldehyde-3-phosphate dehydrogenase. Median is represented by bar.

patients showed highest gene expression for the intermediate-producer genotype at position –1082 and for the low-producer genotype at positions –819 and –592. Highest gene expression in controls was seen for high type at position –1082 and for the low type at positions –819 and –592. None of these tendencies reach significant levels.

No significant differences were seen when TNF-α production was classified according to the different genotypes and their predicted cytokine production pattern (Fig. 3). Slightly higher expression levels were found for the intermediate-producer genotypes when compared to other genotypes in both groups.

DISCUSSION

This is the first study combining cytokine SNP genotyping with measurement of intrarenal cytokine expres-

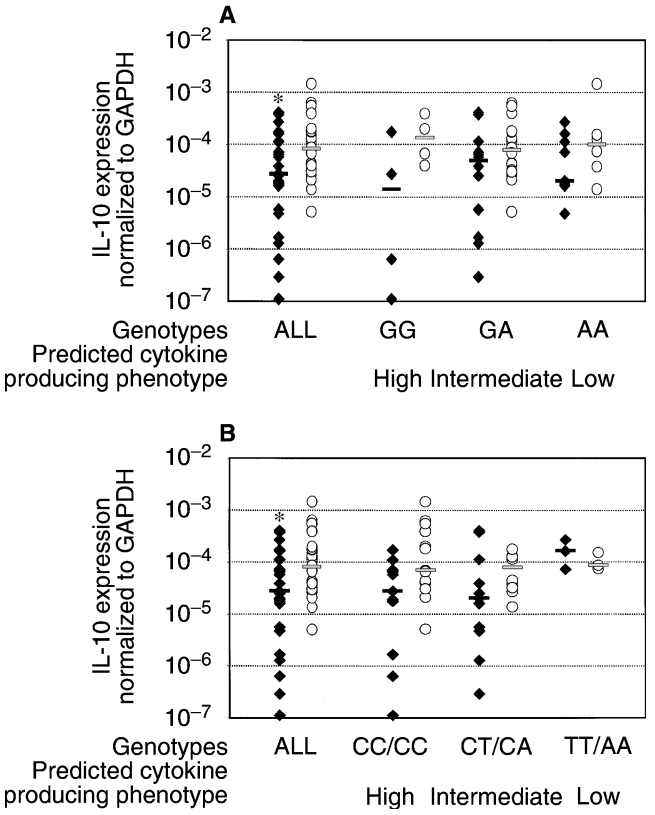

Fig. 2. Intrarenal interleukin-10 (IL-10) mRNA expression for pediatric renal transplant recipients with chronic allograft nephropathy (CAN) (N = 25) (◆) and control individuals (N = 28) (○) for IL-10 position −1082 (*A*) and IL-10 position –819 and –592 (*B*). Overall (ALL) IL-10 expression was different between CAN and controls. $^*P < 0.05$ for the comparison with controls. However, for the different IL-10 genotypes and their predicted producing phenotypes (High, Intermediate, Low). No significant differences were found. GAPDH is glyceraldehyde-3-phosphate dehydrogenase. Median is represented by bar.

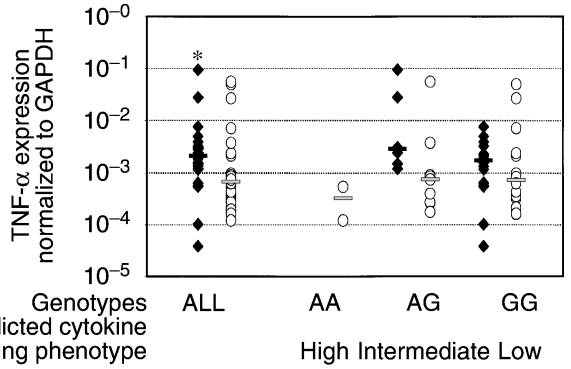

Fig. 3. Intrarenal tumor necrosis factor-α (TNF-α) mRNA expression for pediatric renal transplant recipients with chronic allograft nephropathy (CAN) (N = 25) (◆) and control individuals (N = 28) (○) for position −308. Overall (ALL) TNF-α expression was different between CAN and controls. $^*P < 0.05$ for the comparison with controls. However, for the different TNF-α genotypes and their predicted producing phenotypes (High, Intermediate, Low) no significant differences were found. GAPDH is glyceraldehyde-3-phosphate dehydrogenase. Median is represented by bar.

sion for TGF-β1, IL-10, and TNF-α in renal transplant recipients. In our patient cohort, we were unable to confirm a higher incidence of so-called high-producer cytokine genotypes for any of the investigated cytokine in the CAD patients. Furthermore, when intragraft expression in CAN patients was analyzed based on the genotype, no correlation was found. However, for control specimens there was a correlation for TGF-β1 expression and the underlying genotype with significantly higher TGF-β1 expression in the homozygous so-called high-producing T allele at codon 10.

Distribution of alleles and genotypes in our control group was similar to what had been described by others for TGF-β1 [1, 2] and IL-10 [8, 15, 24, 25]. For TNF-α, we found a higher frequency of the low-producing G allele when compared to other studies in Caucasian population [24–26]. Our numbers are closest to what has been recently published for African Americans and Asians [27] for which the distribution was 78% and 76% for homozygous G, and 18% and 21% for heterozygous A allele, and the G allele frequency was 88% or 89%, respectively. Numbers from Gambia and Japan [28, 29] are higher.

Our two patient groups, stable and CAD, showed no significant differences in their genotypic and allelic distribution when compared to controls. This is in contrast to other groups that suggested an association of long-term kidney graft failure with high TGF-β1 producer genotypes [30]. The fact that there was a tendency toward fewer CAD patients in the IL-10 high producer group for position –819 and –592, however, is consistent to what was described by others [16]. However, since negative findings need to be confirmed in enormous population size, we cannot completely rule out a role of the investigated genotypes in CAN. Based on the differences found in our population (a small effect size of 0.06), a sample size including up to 2700 patients should have been investigated to detect differences with a power of 80% [31].

A biologic reason for the discrepancy to other studies could be that we only included children. It has been suggested that the immune response in children is more reactive when compared to adults [32], which might explain the differences. Therefore, conclusions from our study can only be made for a pediatric population and are not necessarily extendable to adult renal recipients. Thereby, our results do not contradict other opposing findings in adults.

The relationship between cytokine production and cytokine SNPs is based on in vitro studies [1, 7, 9, 10, 18, 19]. We tested whether patients or controls with a so-called high-producing genotype would in fact have higher intrarenal cytokine production. Our CAN patients showed overall higher intragraft mRNA levels for

TGF-β1 and TNF-α and lower intragraft mRNA levels for IL-10 when compared to controls. The findings on TGF-β1 are consistent with earlier publications on CAN reporting higher TGF-β1 levels in serum [33], urine [34], and graft [4, 5, 35]. Superior long-term graft survival in renal transplant patients has been associated with both low [36] as well as high IL-10 levels [16]. The higher levels for TNF-α were unexpected, especially since we carefully excluded infection or acute rejection in this patient cohort.

Analysis of cytokine expression levels by genotypes showed no differences for any of the investigated SNPs in CAN. Interestingly, the same analysis in normal control specimens revealed an association between production phenotype and underlying genotype for codon 10 of TGF-β1. Comparison of overall TGF-β1 expression had shown lower expression for controls vs. CAN. This difference is mainly derived from the difference in expressions for the low-producing phenotype for which the median value in the control group is only a fourth of what is seen in patients. This suggests that this TGF-β1 codon 10 SNP might play a role at a basal state of cytokine production, but its importance may be lost if stimulation occurs as in CAN. One would assume that CAN patients started off with the same genotype-phenotype association at their implant biopsies. This observation could be regarded as a gene-environment interaction, since cytokine expression is probably regulated by other yet unknown genetic and environmental factors. These multifactorial influences on gene expression cannot be understood completely in a unidirectional sense. Therefore, our finding of different cytokine expression patterns in patients and controls should attract further attention if it could be confirmed in larger studies. These studies should also include donor genotypes. No association was found in any of the other SNPs for TGF-β1, IL-10, or TNF-α.

We are aware that our study has limitations. One potential limitation could be patient selection. We chose a cross-sectional approach for which all patients presenting to the outpatient clinic were asked to participate and after follow-up only those with clear stable or CAD were selected. Thus, patients with early graft loss were excluded from the study. However, as our study examined the long-term outcome after transplantation, we do not believe that missing patients with rapid loss of renal function biases our results. The fact that some patients that could have fulfilled CAD criteria and might have lost their graft before entering the study would only have influenced the absolute number but not the characteristics of this patient group. We therefore believe our patient selection did not influence the results substantially.

Ideally, one would like to study protein in addition to mRNA levels, especially for TGF-β1 and TNF-α that are not entirely transcriptionally regulated. However, the amount of tissue available from pediatric biopsies makes this approach unfeasible and gene expression studies have been shown to provide excellent markers to indicate graft response in rejection [37, 38].

The clinical value of cytokine polymorphisms has been previously doubted [39]. Our data, for the first time evaluating corresponding SNP distribution and intragraft gene expression, indicate no relevant genotype-phenotype correlation. We conclude that prospective genotyping for the examined SNPs in pediatric renal transplantation and potential modifications of immunosuppressive strategies based on SNPs are not promising to prevent CAN in children.

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